Defect of hepatocyte growth factor production by fibroblasts in human pulmonary emphysema

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Am J Physiol Lung Cell Mol Physiol 288: L641–L647, 2005. First published December 3, 2004; doi:10.1152/ajplung.00249.2004.—Pulmonary emphysema results from an excessive degradation of lung parenchyma associated with a failure of alveolar repair. Secretion by pulmonary fibroblasts of hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) is crucial to an effective epithelial repair after lung injury. We hypothesized that abnormal HGF or KGF secretion by pulmonary fibroblasts could play a role in the development of emphysema. We measured in vitro production of HGF and KGF by human fibroblasts cultured from emphysematous and normal lung samples. HGF and KGF production was quantified at basal state and after stimulation. Intracellular content of HGF was lower in emphysema (1.52 pg/µg, range of 0.15–7.40 pg/µg) than in control fibroblasts (14.16 pg/µg, range of 2.50–47.62 pg/µg; P = 0.047). HGF production by emphysema fibroblasts (19.3 pg/µg protein, range of 10.4–39.2 pg/µg) was lower than that of controls at baseline (57.5 pg/µg, range of 20.4–116 pg/µg; P = 0.019) and after stimulation with interleukin-1β or prostaglandin E2. Neither retinoic acids (all-trans and 9-cis) nor N-acetylcysteine could reverse this abnormality. KGF production by emphysema fibroblasts (5.3 pg/µg fibroblasts, range of 2.2–9.3 pg/µg) was similar to that of controls at baseline (2.6 pg/µg, range of 1–6.1 pg/µg; P = 0.14) but could not be stimulated with interleukin-1β. A decreased secretion of HGF by pulmonary fibroblasts could contribute to the insufficient alveolar repair in pulmonary emphysema.

The mechanisms of alveolar epithelial repair have so far been described in the context of acute lung injury and lie mainly in the ability of type 2 pneumocytes to proliferate, migrate along the denuded basal membrane, and eventually differentiate into type 1 pneumocytes (24). The role of mesenchymal-epithelial interactions in this process is essential. Pulmonary fibroblasts have the ability to produce and maintain the extracellular matrix structure, whose integrity is needed for epithelial cells to migrate and differentiate (26). Furthermore, fibroblasts produce and release several growth factors that stimulate alveolar epithelial cell proliferation, differentiation, and migration, such as keratinocyte growth factor (KGF) (also known as the fibroblast growth factor-7) and hepatocyte growth factor (HGF). Both molecules facilitate the repair of an experimental epithelial wound in vitro (11, 12) and are protective in a number of animal models of acute lung injury (34). Moreover, besides their potent action on alveolar epithelial cells, KGF and HGF have been shown to act on endothelial cells, as both factors promote endothelial cell survival and favor angiogenesis (4, 13). Production of HGF and KGF is part of the normal response to lung injury, as evidenced in animals (1) and in humans (31). In premature infants, a low KGF concentration in the airways is associated with the further development of bronchopulmonary dysplasia (9). Interestingly, a link has been evidenced between retinoids and KGF and HGF because ATRA may regulate KGF and HGF expression in vitro (6, 21) and in vivo (20). Recently, HGF has been shown to orchestrate pulmonary regeneration in emphysematous lung in mice (15).

Our hypothesis is that altered properties of lung fibroblasts contribute to defective alveolar repair in pulmonary emphysema. This question has not been previously evaluated, and very limited data concerning the functional properties of pulmonary fibroblasts in emphysema are available. In vitro proliferative capacity of lung fibroblasts has been shown to be reduced in patients with emphysema compared with controls, a finding that may negatively influence the alveolar repair process (28). No data are available concerning the production of KGF and HGF, essential mediators of alveolar repair, by lung fibroblasts in emphysema.

Therefore, the aim of this study was to evaluate the production of HGF and KGF by human lung fibroblasts cultured from normal and emphysematous lungs and to determine the modulatory action of retinoids.

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MATERIALS AND METHODS

This study was approved by the ethics committee of Paris-Bichat University Hospital. Informed consent was obtained from patients.

Study Population

Pulmonary emphysema patients. Fibroblasts were cultured from lung samples from nine patients (7 men) with severe pulmonary emphysema who were undergoing lung volume reduction surgery \((n = 5)\) or lung transplantation \((n = 4)\). Median age of patients was 60 yr (range of 41–70 yr). All patients were smokers or ex-smokers \((34 ± 14 \text{ pack-yr})\), with the exception of one patient who had α1-proteinase inhibitor deficiency. Emphysema was diagnosed by the presence of an obstructive ventilatory disorder via lung function tests associated with characteristic chest computed tomography and histological findings. The absence of any associated lung disease was verified. Five patients received inhaled steroids at the time of sampling.

Control patients. Fibroblasts were cultured from lung samples from eight patients (5 men) undergoing lung surgery for cancer \((n = 6)\), foreign body removal \((n = 1)\), or pleuradesis \((n = 1)\). Median age of controls was 61 yr (range of 41–67 yr). Lung samples were taken from an uninvolved segment, and the absence of emphysema was histologically controlled. Four patients were active or past smokers \((30 ± 1 \text{ pack-yr})\), and four never smoked. One patient received inhaled steroids for asthma treatment.

Culture of Fibroblasts

Pulmonary fibroblasts were cultured from lung explants as previously described (23). Fibroblasts were cultured with DMEM culture medium (GIBCO/Invitrogen, Cergy-Pontoise, France) with 10% fetal calf serum (Fetalclone 2, Hyclone, Logan, UT), 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (GIBCO/Invitrogen). Cells were maintained at 37°C with 5% CO2 and 100% humidity in an atmosphere of 95% air and 5% CO2 in a humidiﬁed incubator. Fractalkine (MIP-3α, R&D Systems) was added to the medium to ensure continuous production and secretion of IL-1β.

Fibroblasts were cultured to confluence in 75-cm² flasks and deprived of serum for 18 h. The culture medium was aspirated, the cells were rinsed twice with PBS, and then the cell monolayer was lysed with the CytoBuster protein extraction reagent (Novagen, Darmstadt, Germany). HGF and KGF were quantified in the lysate with the Duoset ELISA, as described above. The total protein content of the cell monolayer was determined with the Bio-Rad protein assay reagent (Bio-Rad, Marne-la-coquette, France). HGF secretion and KGF secretion by fibroblasts were expressed as picograms HGF or KGF per microgram of protein in the cell monolayer.

Measurement of HGF and KGF Concentration

HGF and KGF concentrations in cell supernatants were measured with HGF and KGF ELISA (Duoset development kits, R&D, Abingdon, UK). Sensitivity of the tests was 125 pg/ml for HGF and 15 pg/ml for KGF. Total cellular proteins were recovered in Tris-HCl after two freeze-thaw cycles and sonication, and the protein content of the cell monolayer was determined with the Bio-Rad protein assay reagent (Bio-Rad, Marne-la-coquette, France). HGF secretion and KGF secretion by fibroblasts were expressed as picograms HGF or KGF per microgram of protein in the cell monolayer.

Determination of Intracellular HGF and KGF

Fibroblasts were grown to confluence in 75-cm² tissue culture plates (Corning, Schiphol-Rijk, The Netherlands), washed twice in PBS (GIBCO/Invitrogen), then incubated in serum-free DMEM, and exposed to stimulants or kept unstimulated. For HGF secretion determination, cells were incubated for 18 h in 1 ml of DMEM. For KGF secretion determination, cells were cultured for 48 h in 500 μl of DMEM because, in preliminary experiments, KGF concentrations were below the detection level after an 18-h incubation period in 1 ml of DMEM. Cell supernatants were stored at −80°C until determination of HGF and KGF concentrations. Fibroblasts were stimulated with interleukin-1β (IL-1β, 10 ng/ml) or prostaglandin E2 (PGE2, 10−6 M) at concentrations previously shown to exert a maximal stimulatory effect on HGF and KGF secretion by fibroblasts (23, 32). N-acetylcysteine (NAC) is a potent anti-oxidant agent proven to inhibit oxidative stress and apoptosis in human lung fibroblasts exposed to cigarette smoke extract in vitro (5) and to be protective in animal models of emphysema (2, 29). In some experiments, control and emphysema fibroblasts were cultured with 10−2 M NAC to determine whether this agent could modulate the secretion of HGF and KGF by those cells. To appreciate the regulatory effect of retinoids, fibroblasts were cultured with 10−6 M ATRA or 9-cis-retinoic acid (9cisRA, 10−6 M). For these experiments, cells cultured with 1% DMSO were used as controls because ATRA and 9cisRA were dissolved in DMSO. All stimulants were obtained from Sigma (Saint-Quentin Fallavier, France).

Quantitative Analysis of proHGF and KGF mRNA

Fibroblasts were grown to confluence in 75-cm² tissue culture flasks and cultured in serum-free DMEM for 18 h. Total RNA was extracted and reverse transcribed as described previously (23). Quantitative real-time PCR using a Sybr green fluorochrome (Sigma) was performed on an ABI Prism 7700 cycler (AME Bioscience, London, UK) to quantify proHGF mRNA (proHGF is the inactive precursor of hepatocyte growth factor). Primers sequences and amplification products are described in Table 1.

Statistical Analysis

All data are expressed as medians (with ranges given in parentheses). Differences between emphysema and control fibroblasts were determined with the Wilcoxon-Mann-Whitney non-parametric test.

Table 1. Quantitative PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Amplification Product</th>
</tr>
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<tbody>
<tr>
<td>proHGF</td>
<td>Forward</td>
<td>5’-CACAGGGGACAAAAAGAAAGAA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCAAGGAAGATGGGAACGTTTTA-3’</td>
</tr>
<tr>
<td>KGF</td>
<td>Forward</td>
<td>5’-GACAAAGGAAAGAAACCTATGCAA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-AAGTGCGTCCTTCTCTTCTC-3’</td>
</tr>
<tr>
<td>Ubiquitin C</td>
<td>Forward</td>
<td>5’-CACCTGCGCCCTGCGTTGCA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TTTTTCTGCGGAGAATGCG-3’</td>
</tr>
</tbody>
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KGF, keratinocyte growth factor; proHGF, inactive precursor of hepatocyte growth factor.
detected by the Mann-Whitney U-test. To compare the effect of pharmacological agents on baseline conditions, we used Friedman’s ANOVA test, which was followed by Wilcoxon’s paired test for group comparisons. Comparisons were made with Spearman’s non-parametric correlations test for continuous variables and with Mann Whitney’s U-test for categorical variables. A P value of <0.05 was considered significant.

RESULTS

HGF Secretion by Unstimulated Fibroblasts

All pulmonary fibroblasts from control subjects secreted detectable levels of HGF in vitro [57.5 pg/μg (20.4–116 pg/μg)]. There was no observable difference between smokers or ex-smokers and those who never smoked.

Nine emphysema fibroblast cultures were evaluated for HGF secretion. HGF concentrations were below the detection level of the assay for three of them. For statistical analysis, a value of 125 pg/ml was attributed to these cell lines. Unstimulated HGF secretion by emphysema fibroblasts was markedly lower than that of controls (P = 0.019; Fig. 1). HGF secretion did not correlate with age, forced expiratory volume in 1 s, residual volume, total lung capacity, carbon monoxide diffusing capacity, or tobacco smoke exposure expressed as pack-years of smoking.

Intracellular HGF was determined in five emphysema and five control fibroblast cultures. The intracellular HGF content of emphysema fibroblasts [1.52 pg/μg (0.15–7.40 pg/μg); P = 0.047] was lower than that of control fibroblasts [14.16 pg/μg (2.50–47.62 pg/μg)]. Intracellular HGF did not correlate with HGF secretion in control and emphysema fibroblasts (Fig. 1).

ProHGF mRNA was detected in all lung fibroblast cultures. ProHGF-to-ubiquitin C mRNA ratio in unstimulated emphysema fibroblasts [0.511 (0.053–2.29)] was lower than that of control fibroblasts [1.76 (0.76–37.59); P = 0.021; Fig. 1].

Modulation of HGF Secretion

HGF secretion by control fibroblasts was increased by IL-1β [131.5 pg/μg (50.7–291.3 pg/μg); P = 0.011] and PGE2 [238 pg/μg (88.2–428.1 pg/μg); P = 0.011] (Fig. 2). Stimulation by PGE2 was more potent than that induced by IL-1β (P = 0.017) (Fig. 2). HGF secretion by control fibroblasts cultured with DMSO was 35.4 pg/μg (8.6–102.5 pg/μg). ATRA [35.6 pg/μg (7.6–184.3 pg/μg)] and 9cisRA [28.2 pg/μg (8.1–128.8 pg/μg)] did not significantly modulate HGF secretion by control fibroblasts (P = 0.67 and 0.16, respectively).

HGF secretion by emphysema fibroblasts was increased by IL-1β [35.6 pg/μg (6.1–70.2 pg/μg); P = 0.017] and PGE2 [48.9 pg/μg (22.3–66.1 pg/μg); P = 0.018, Fig. 3] and reached levels similar to that of unstimulated controls. HGF secretion by emphysema fibroblasts cultured with DMSO was 16.8 pg/μg (2.6–36.2 pg/μg). ATRA [11 pg/μg (0–33 pg/μg)] and 9cisRA [14.9 pg/μg (1.7–57.3 pg/μg)] did not modulate HGF secretion by emphysema fibroblasts (P = 0.21 and 0.31, respectively). The effect of NAC (10⁻² M) on HGF secretion was evaluated in five emphysema fibroblast cell lines. In these experiments, NAC inhibited HGF secretion [unstimulated: 26.0 pg/μg (16.3–39.2 pg/μg); NAC: 14.6 pg/μg (7.1–25.46 pg/μg); P = 0.04, Fig. 3].

KGF Secretion by Unstimulated Fibroblasts

Seven control fibroblast cultures were evaluated for KGF secretion (see Fig. 5). All produced detectable amounts of KGF at baseline in vitro [2.6 pg/μg (1–6.1 pg/μg)]. Unstimulated secretion of KGF by fibroblasts from smokers [4.45 pg/μg...
basal condition or exposed to 10 ng/ml IL-1β or 10⁻⁶ M PGE₂. Individual values and median (bar) are shown.

(2.6–6.1 pg/µg); n = 3] was higher than that of nonsmokers [1.4 pg/µg (1.2–5 pg/µg); n = 4, P = 0.033].

Seven emphysema fibroblast cultures were evaluated for KGF secretion in vitro (see Fig. 6). KGF secretion by emphysema fibroblasts [5.3 pg/µg (2.2–9.3 pg/µg)] was similar to that of controls (P = 0.14; Fig. 4).

Intracellular KGF was determined in five emphysema and five control fibroblast cultures. The KGF content of control fibroblasts [1.58 pg/µg (0.77–3.8 pg/µg)] was not different from that of emphysema fibroblasts [0.99 pg/µg (0.4–2.16 pg/µg); P = 0.17].

KGF mRNA was detected in all lung fibroblast cultures. KGF-to-ubiquitin C mRNA ratio in emphysema fibroblasts [1.014 (0.44–20.23)] was not different from that of control fibroblasts [2.36 (0.66–14.08); P = 0.44; Fig. 4].

Modulation of KGF Secretion

KGF secretion by control fibroblasts was increased by IL-1β [4.6 pg/µg (3.4–12 pg/µg); P = 0.042] and PGE₂ [3.8 pg/µg (1.7–11.9 pg/µg); P = 0.028] (Fig. 5). By contrast, IL-1β did not stimulate KGF secretion by emphysema fibroblasts [7.3 pg/µg (2.1–10.1 pg/µg); P = 0.86], whereas the stimulatory effect of PGE₂ was maintained [9.8 pg/µg (4.1–21.5 pg/µg); P = 0.027] (Fig. 6).

KGF secretion by control fibroblasts cultured with DMSO was 3.6 pg/µg (1.1–8.9 pg/µg). KGF secretion by control fibroblasts cultured with ATRA [2.7 pg/µg (0.9–4.7 pg/µg)] was not different from that of cells cultured with DMSO (P = 0.09), whereas 9cisRA had an inhibitory effect [1.7 pg/µg (0.9–5.1 pg/µg); P = 0.034]. KGF secretion by emphysema fibroblasts cultured with DMSO was 3.2 pg/µg (0–23 pg/µg). ATRA [4.1 pg/µg (0–15.9 pg/µg)] and 9cisRA [3 pg/µg (1.2–12.4 pg/µg)] did not modulate KGF secretion by emphysema fibroblasts (P = 0.17 and 0.46, respectively).

**DISCUSSION**

The main findings of this study are 1) that lung fibroblasts cultured in vitro from patients with pulmonary emphysema have a decreased ability to produce and to secrete HGF compared with those cultured from control patients and 2) that KGF secretion by emphysema fibroblasts is similar to that of controls at baseline but is not stimulated by IL-1β.

Fibroblasts play an important role in lung development and homeostasis and are involved in the complex repair process that follows lung injury. We studied lung fibroblasts from patients with severe emphysema requiring lung volume reduction surgery or transplantation. Pulmonary fibroblasts were obtained by primary culture of tissue fragments and were studied at the fifth passage, after prolonged cultivation. Their functional properties may have been quite different from those of in vivo cells. However, because control and emphysema fibroblasts were similarly cultured, this is unlikely to explain the difference between cell types. Considering the heterogeneous repartition of lesions in pulmonary emphysema, it is possible that culture of lung samples from mildly emphysematous lung areas may have led to different results. This could not be done in our study. However, we studied fibroblasts from smokers without emphysema to control for the possible role of tobacco exposure. Every other control patient was an active or past smoker, and, although none had detectable lung emphy-
It remains difficult to exclude the presence of minimal emphysema at histological examination of frozen and poorly inflated lung samples obtained from resected specimens. We observed that HGF secretion by unstimulated emphysema pulmonary fibroblasts was two to three times lower than that of controls. To assess whether a decreased HGF secretion by emphysema fibroblasts was related to a decreased production of HGF by those cells, we determined the intracellular content of HGF in emphysema and control fibroblasts. Intracellular HGF was markedly lower in emphysema fibroblasts than in control fibroblasts. This points to a defect in HGF production by those cells. This reduction in HGF production and secretion by emphysema fibroblasts was associated with a reduction of proHGF mRNA content compared with control fibroblasts. NAC, a potent antioxidant molecule, did not increase and eventually had an inhibitory effect on HGF production by emphysema fibroblasts. It is therefore unlikely that the lower HGF secretion by those cells was related to oxidative stress, which is known to participate in the pathogenesis of emphysema (22). HGF secretion by emphysema fibroblasts could still be stimulated by IL-1β and PGE2, demonstrating that the regulatory machinery of HGF secretion was functional in these cells. In preliminary experiments, we verified that 10^{-2} M NAC had no cytotoxic effects on control and emphysema fibroblasts.

The defect of HGF production and secretion that we measured was not the consequence of a generalized modification of...
gene expression and protein production and secretion by emphysema fibroblasts but appeared to be specific for HGF. Indeed, unstimulated KGF protein content and secretion and mRNA transcription by emphysema fibroblasts were similar to results shown in controls.

Besides fibroblasts, different lung cell types have been shown to produce HGF, such as smooth muscle cells (27), neutrophils (14, 16), and perhaps alveolar macrophages, although this remains debatable (8). Neutrophils are increased in the lung in emphysema (10) and could contribute to HGF secretion in emphysema in vivo. At this time, there are no data concerning HGF expression in situ in the emphysematous lung.

KGF secretion by emphysema fibroblasts was not stimulated by IL-1β, whereas IL-1β did stimulate HGF secretion in those cells. This discrepancy may indicate that the stimulation by IL-1β of KGF and HGF secretion by lung fibroblasts involves separate signaling pathways downstream of the IL-1 receptor. Indeed, many intracellular signal transduction pathways have been shown to be activated by the binding of IL-1 to its receptor (3). A defect in the stimulatory effect of IL-1β on KGF secretion may lead to a relative deficiency of KGF secretion by fibroblasts in the context of alveolar injury and contribute to a defective alveolar repair.

KGF and HGF are survival and growth factors for epithelial and endothelial cells and stimulate angiogenesis (4, 13). A defect in KGF or HGF production by neighboring fibroblasts could contribute to the excessive apoptosis of epithelial and endothelial cells, which is now recognized as an important step in the constitution of emphysema. Underexpression of vascular endothelial growth factor, a potent mitogen for endothelial cells and a strong promoter of angiogenesis, has been recently shown to be involved in the pathophysiology of pulmonary emphysema in animals and in humans (17, 18). Whether underexpression of HGF or KGF favors the development of emphysema in vivo has not been previously evaluated in the literature and warrants further study. Recently, Ishizawa et al. (15) showed that intraperitoneal recombinant human HGF abrogates elastase-induced emphysema in mice through the mobilization of endothelial precursors cells and their engraftment in the damaged alveoli.

Interestingly, we previously showed that fibroblasts from idiopathic pulmonary fibrosis lungs secrete low levels of HGF and contain lower amounts of proHGF mRNA than control fibroblasts (23). Alveolar repair is thought to play an important role in the pathogenesis of IPF as well as emphysema (30, 33). A defect in HGF secretion by fibroblasts could contribute to this phenomenon in both diseases.

ATRA has been shown to promote alveolar repair in elastase-induced emphysema in rats (25). Retinoids have been shown to modulate HGF and KGF secretion in different human cell types (6, 21). However, neither ATRA nor 9cisRA (10⁻⁶ M) was able to regulate positively HGF secretion by emphysema or control fibroblasts in vitro in our study. In contrast, 9cisRA had an inhibitory effect on KGF secretion by control fibroblasts instead of the expected stimulatory effect. Interestingly, 9cisRA did not modulate KGF secretion by emphysema fibroblasts. Thus the beneficial role of ATRA demonstrated in animal models of pulmonary emphysema is unlikely to be secondary to an effect on HGF or KGF secretion by lung fibroblasts.

Altogether, our results indicate that pulmonary emphysema fibroblasts have a reduced capacity to produce and secrete HGF in vitro compared with control fibroblasts. This may contribute to the insufficient alveolar repair observed in pulmonary emphysema.

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